On-column Chemical Refolding of Proteins

The development of a protocol for the efficient refolding of the proteins from inclusion bodies has become an important issue because it can provide soluble native protein for structural and functional studies. Here we report on an on-column refolding method, established at the Berkeley Structural Genomics Center, for use in protein structure determination. It is based upon an "artificial chaperone-assisted refolding" method in solution that was proposed previously but has been modified to provide all the advantages of a chromatographic procedure and provides the additional benefit of protein purification.

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During the past several years, more than 25 structural genomics projects from different countries have been organized to evaluate large numbers of candidate targets (1). They aim to determine the structure of representatives of particular protein families and lay a foundation for a global understanding of architecture, function and fold evolution of proteins (2). Due to fast growth, easy handling and low cost, *Escherichia coli* is the principal expression system of choice for most structural genomic projects. One of the biggest problems, however, is expressing the proteins in a soluble form — a necessary step for crystallization and x-ray crystallographic study. Often recombinant proteins produced in *E. coli* or other heterologous expression systems accumulate as insoluble aggregates, known as inclusion bodies.

Conventional methods for refolding insoluble recombinant proteins include slow dialysis or dilution into a large volume of refolding buffer or chromatographic refolding using packed columns. Chromatographic methods can include solvent-exchange size exclusion chromatography and immobilization of the denatured protein onto a column or gel matrix, with subsequent dilution of denaturant to promote refolding. A large amount of data in the literature provides information aimed at enhancing the refolding yield of inclusion body proteins by adding certain low-molecular-weight additives to reduce protein aggregation. Surfactants and detergents have proven to be efficient folding aids and have been shown to work with a variety of proteins in dilution or chromatographic refolding (3–6). One drawback to their use, however, is that they are difficult to remove — a direct result of their ability to bind to proteins and to form micelles. Rozema and Gellman (7) developed a new dilution-based folding strategy in which the denatured protein first is exposed to a detergent-containing solution to prevent aggregation, followed by stripping of the detergent with cyclodextrin in a large volume of buffer to promote refolding. This method has been claimed to mimic the GroEL–GroES chaperonin action *in vivo* and has been called "artificial chaperone-assisted refolding;" several proteins have been refolded successfully by this method (8–10). Although this process is attractive due to its efficacy and practicality, it is time-consuming and accompanied by loss of the protein during filtration and concentration of large volumes.

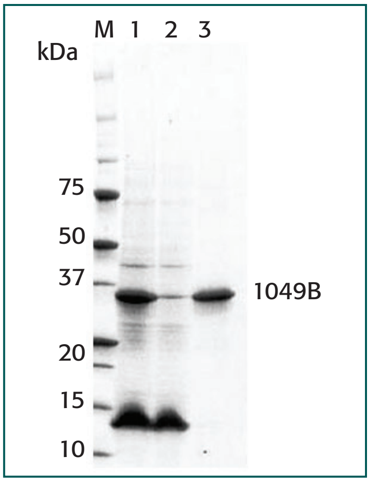
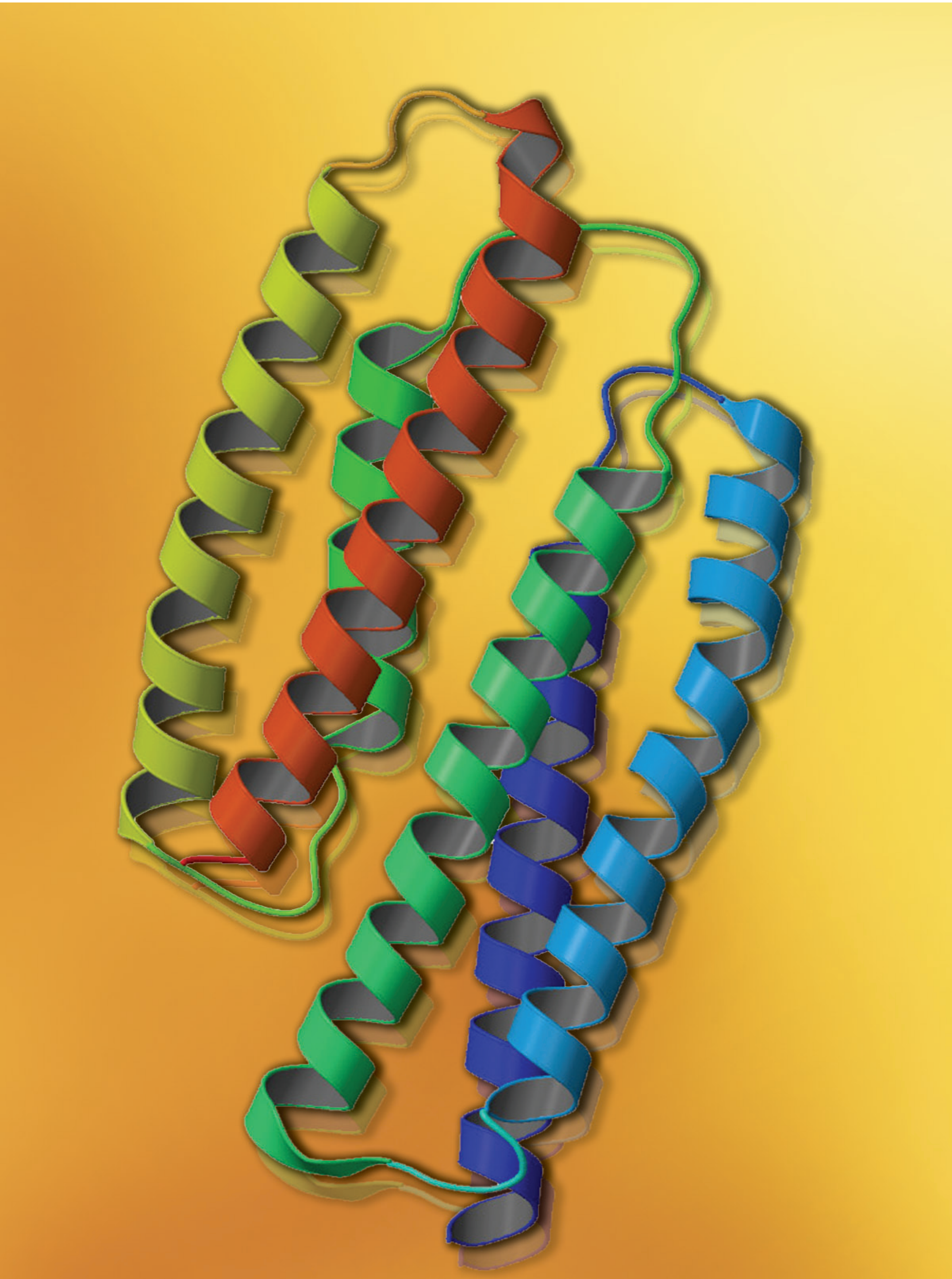


Figure 1. SDS-PAGE analysis of a BSGC target (1049B, MW = 36 kDa) that was purified and refolded by the on-column chemical refolding method. M, protein MW marker; Lane 1, IB solubilized in 8 M urea before applying onto Ni-NTA column; Lane 2, proteins in the Ni-NTA flow through fraction; Lane 3, eluted fraction (300 mM imidazole) of refolded protein.

Here we describe a refolding method for insoluble His-tagged proteins expressed in *E. coli*, modified from the "artificial chaperone-assisted" method. Inclusion bodies solubilized in urea first are bound to an affinity column and exposed to a detergent wash to prevent misfolding. This is followed by a β-cyclodextrin wash that removes the detergent and promotes correct folding. The target protein is eluted with imidazole, goes through further purification steps (ion exchange chromatography [IEX] and/or size exclusion chromatography [SEC]) and is evaluated by dynamic light scattering detection. We have been able to obtain 30–100% of the proteins refolded in seven out of 10 tested proteins. Six of the seven refolded proteins were able to produce crystals of varying qualities.

Experimental Conditions

The proteins that we have worked on are expressed from genes from *Mycoplasma pneumoniae*, *Mycoplasma genitalium* or their



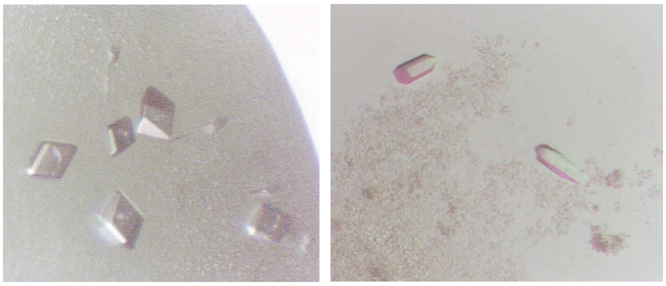


Figure 2. Crystals obtained from two BSGC targets refolded by on-column chemical refolding. A) Refolded 1105B was crystallized using the vapor diffusion method at room temperature. The optimum conditions were found in 0.1 M Tris-HCl, pH 8.5, 0.2 M Li_2SO_4, 30% (w/v) PEG 4K. The crystal dimensions were 200 × 200 × 50 microns. B) Refolded 1349B was crystallized using the vapor diffusion method at room temperature. The optimum conditions were found in 100 mM MES, pH 5.8, 0.5 M NaCl. The crystal dimensions were 45 3 50 3 30 microns.

homologs from other organisms. The protein identification numbers listed in Table I are targets from the Berkeley Structural Genomics Center (BSGC, Berkeley, California, USA, www.strgen.org). The His-tagged target proteins were expressed in *E. coli* as inclusion bodies and collected as a pellet after centrifugation of the disrupted cells. Inclusion body solubilization was carried out in 8-M urea or 6-M guanidinium hydrochloride. Solubilized inclusion bodies were bound to Ni-NTA resin (Qiagen, Valencia, California, USA) and pre-equilibrated in denaturing buffer — by batch-absorption performed overnight at room temperature. The amount of resin used was calculated according to the manufacturer's recommendation (5 mg of protein/1 ml of resin). On-column renaturation and purification were performed the next morning by several changes of buffers. First, the column was washed using the denaturing buffer containing 20 mM imidazole to remove nonspecifically-bound contaminants. β-mercaptoethanol at a final concentration of 10 mM was added to this buffer and all following buffers if the refolding protein contained cysteines. All renaturation steps were carried out in Buffer A (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl). The pH of Buffer A is dependent upon the pI of the target protein.

In the next step, the column was washed with 10-column volumes (CV) of buffer containing 0.1% Triton X-100 (Anatrace, Maumee, Ohio, USA). This was followed by a wash with 10 CV of Buffer A containing 5 mM β-cyclodextrin (Sigma, St. Louis, Missouri, USA) to remove detergent from the protein–detergent complex and to allow the protein to refold. Before elution, an additional wash with 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl was applied to remove remaining impurities and β-cyclodextrin. Refolded protein was eluted with Buffer A that was supplemented with 300–600 mM imidazole. The eluted fractions containing soluble refolded protein were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to further purification steps (IEX and/or SEC).

Results and Discussion

To obtain pure soluble protein from inclusion bodies for crystallographic purposes, we developed a new refolding method by using a column-based approach with detergent and cyclodextrin as low-molecular-weight additives. Refolding in the presence of a detergent followed by addition of cyclodextrin has been pioneered by Rozema and Gellman (7). Their method utilizes a dilution approach, whereby a protein denatured by urea or guanidinium-HCl is diluted to a low concentration in a large volume of detergent-containing buffer, then diluted again in the presence of a cyclodextrin solution. After refolding is completed, the protein solution is passed through a 0.22-µm filter to remove aggregated protein, and then through a 10 kDa cutoff filter to remove detergent and cyclodextrin. In order to adapt the procedure for our purposes, we altered the original method and applied it to His-tagged proteins expressed as inclusion bodies in *E. coli*. Table II compares, step by step, the method by Rozema and Gellman (7) and our on-column refolding method. The advantages of on-column chemical refolding include lack of dependence upon protein concentration, high yields of soluble protein, simultaneous purification and refolding and amenability to high-throughput refolding. The effectiveness of this refolding method has been confirmed by applying it to different BSGC targets. We have been able to obtain 30–100% of the proteins refolded in seven out of 10 tested proteins (Table I). Dynamic light scattering was performed on all the samples in Table I to determine their monodispersity. Out of the six samples that could be refolded, five were monodisperse (radius: <5 nm; polydispersity: <30%). Figure 1 shows an SDS-PAGE analysis of one of the refolded proteins. Six of the seven refolded proteins were able to produce crystals of varying qualities, and Figure 2 shows two of these crystals. The crystal structure of 1105B did not show any presence of detergent.

Conclusion

One of the key steps in structural genomics is the rapid production of purified native protein. The expression of recombinant proteins in transformed microorganisms often is hampered by the formation of insoluble protein aggregates. Technology for refolding proteins that are expressed as inclusion bodies still is a major bottleneck in protein production. Such technology must be scalable, easily automated, applicable for a broad range of proteins and economical. On-column chemical refolding meets these criteria. Column-based refolding with detergent

Table I. Summary of BSGC protein targets refolded by an on-column chemical refolding method.

Targets	MW, kDa	% "Refolded" *	Crystallized
1049B	36	50	Yes
1084B	40	40	Yes
1089B	17	100	No
1105B	70	100	3.2 A data/solved
1113B	19	100	Yes
1277B	44	100	Yes
1294B	49	0	Not applicable
1315B	61	0	Not applicable
1338B	16	0	Not applicable
1349B	20	100	2.8 A data

*Percent target eluted/percent target loaded

Table II. Dilution- and column-based refolding methods using detergent and β-cyclodextrin as additives.

Artificial chaperone-assisted refolding (7)	On-column chemical refolding
IB solubilization in 8 M urea/ 6 M GdHCl	IB solubilization in 8 M urea/ 6 M GdHCl
Dilution into a large volume of buffer with detergent Dilution with β-cyclodextrin buffer	Binding to Ni-NTA, wash with buffer containing detergent Wash with buffer containing β-cyclodextrin
Filtration (0.2 micron)	
Concentration	Elution with imidazole
Time required	
2–3 days	20 hours

and cyclodextrin promises to facilitate the rapid and efficient refolding of various His-tagged recombinant proteins. The binding of denatured protein through a His-tag followed by a detergent wash efficiently prevents irreversible protein aggregation upon denaturant removal and significantly increases renaturation yield; it also eliminates the process of concentrating a large volume of protein solution at a low concentration — a significant time-consuming step in the dilution-based approach.

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